Azithromycin and clarithromycin inhibit lipopolysaccharide-induced murine pulmonary neutrophilia mainly through effects on macrophage-derived GM-CSF and IL-1β

Martina Bosnar, Berislav Bošnjak, Snježana Čužić, Boška Hrvačić, Nikola Marjanović, Ines Glojnarić, Ognjen Čulić, Michael J. Parnham, Vesna Eraković Haber

GlaxoSmithKline Research Centre Zagreb Limited, Prilaz baruna Filipovića 29, HR-10000 Zagreb, Croatia
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Corresponding author:

Martina Bosnar,  
GlaxoSmithKline Research Centre Zagreb Limited,  
Prilaz baruna Filipovića 29,  
HR-10000 Zagreb, Croatia  
Tel. + 385 (0) 1 6051113  
Fax. + 385 (0) 1 6051019  
Email address: martina.z.bosnar@gsk.com

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Abbreviations: BALF, bronchoalveolar lavage fluid; BEBM, bronchial epithelial basal medium; CF, cystic fibrosis; COPD, chronic obstructive lung disease; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethlysulfoxide; DPB, diffuse panbronchiolitis; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; GM-CSF, granulocyte-macrophage colony stimulating factor; GRO, growth regulated oncogene-alpha; ICAM-1,
intracellular adhesion molecule 1; IL, interleukin; LPS, lipopolysaccharide; macrolides, macrolide antibiotics; MPO, myeloperoxidase; PBS, phosphate buffered saline; TNF-α, tumor necrosis factor alpha; VCAM-1, vascular adhesion molecule 1

**Recommended section:** Inflammation, Immunopharmacology, and Asthma
Abstract

Macrolide antibiotics possess immunomodulatory / anti-inflammatory properties. These properties are considered fundamental for the efficacy of macrolide antibiotics in the treatment of chronic inflammatory diseases like diffuse panbronchiolitis and cystic fibrosis. However, the molecular mechanisms and cellular targets of anti-inflammatory / immunomodulatory macrolide activity are still not fully understood. In order to describe anti-inflammatory effects of macrolides in more detail and to identify potential biomarkers of their activity, we have investigated the influence of azithromycin and clarithromycin on the inflammatory cascade leading to neutrophil infiltration into lungs after intranasal lipopolysaccharide challenge in mice. Azithromycin and clarithromycin pretreatment reduced total cell and neutrophil numbers in bronchoalveolar lavage fluid as well as myeloperoxidase concentration in lung tissue. Additionally, concentrations of several inflammatory mediators including CCL-2, GM-CSF, IL-1β, TNF-α and sE-selectin in lung homogenates were decreased following macrolide treatment. Inhibition of cytokine production observed in vivo was also corroborated in vitro in LPS-stimulated monocytes / macrophages, but not in an epithelial cell line. In summary, results presented in this paper confirm that macrolides can suppress neutrophil-dominated pulmonary inflammation and suggest that the effect is mediated through inhibition of GM-CSF and IL-1β production by alveolar macrophages. Besides GM-CSF and IL-1β, CCL2 and sE-selectin are also identified as potential biomarkers of macrolide anti-inflammatory activity in the lungs.
Introduction

Macrolide antibiotics (‘macrolides’) are a well-established class of antimicrobial agents characterized by the presence of a highly substituted macrocyclic lactone ring. Erythromycin, a natural product isolated from *Saccharopolyspora erythraea*, was the first macrolide to be introduced to clinical use over 50 years ago. Afterwards, several semisynthetic derivatives of erythromycin, like clarithromycin (6-O-methylerythromycin A, CAS 81103-11-9) and azithromycin (9-deoxy-9a-aza-9a-methyl-9a-homoerythromycin A, CAS 83905-01-5), were designed to broaden the antimicrobial spectrum, reduce gastrointestinal side effects and increase acid-stability and bioavailability in this class of antibiotics (Whitman and Tunkel, 1992). Nowadays, macrolides are widely used in the treatment of respiratory tract and soft tissue infections.

In addition to their efficacy in treatment of bacterial infections, many studies over the last 20 years have demonstrated that macrolides are effective in the treatment of various chronic inflammatory disorders of the respiratory tract. Introduction of erythromycin to the treatment of diffuse panbronchiolitis (DPB) in the 1980s drastically increased 10-year survival rate, decreased frequency of exacerbations and restored lung function (Kudoh, et al., 1998). Afterwards, macrolides were successfully used in the treatment of cystic fibrosis (CF), which shares a number of similarities in clinical and pathologic characteristics with DPB. In CF patients, macrolide treatment was shown to significantly improve lung function and reduce frequency of exacerbations. Consequently, macrolides are now first line therapy for DPB and recommended for patients with CF, and they are also being evaluated in the therapy of chronic obstructive lung disease (COPD), chronic sinusitis, asthma, bronchiectasis and bronchiolitis obliterans (reviewed in Crosbie and Woodhead, 2009).

Beneficial effects of macrolide treatment in chronic inflammatory lung diseases cannot be attributed solely to their antimicrobial activity, as the doses used are lower than those
used in standard antibiotic therapy and thus below the minimum inhibitory concentration for the most common respiratory pathogens. In addition, numerous *in vitro* and *in vivo* studies have shown that macrolides also possess a number of anti-inflammatory and immunomodulatory properties. They were shown to inhibit expression of pro-inflammatory cytokines including TNF-α, IL-1β and IL-6, chemokines and adhesion molecules. Moreover, macrolides inhibit recruitment and migration of inflammatory cells and mucus hypersecretion (reviewed in Culic, et al., 2001; Shinkai, et al., 2008). Each or a combination of these anti-inflammatory or immunomodulatory properties could, therefore, account for the efficacy of macrolides in chronic inflammatory lung diseases.

Development of novel macrolide compounds that have anti-inflammatory properties, but are devoid of antimicrobial activity, has been hindered by the lack of knowledge of molecular mechanisms and cellular targets of their anti-inflammatory / immunomodulatory activity. As several groups, including ours, have reported that various macrolides inhibit the lipopolysaccharide-induced pulmonary inflammatory response (Ivetic, et al., 2006; Kadota, et al., 1993; Leiva, et al., 2008; Ou, et al., 2008; Sanz, et al., 2005; Tamaoki, et al., 1995), we have used this model to perform in-depth investigation of the azithromycin and clarithromycin effect on the inflammatory cascade leading to neutrophil infiltration into lungs following intranasal lipopolysaccharide (LPS) challenge in mice. The effect of macrolides on various cytokines, chemokines and adhesion molecules induced by LPS challenge was carefully assessed in order to identify potential biomarkers of their anti-inflammatory activity. The data gathered from the model allowed us to establish a simple *in vitro* test by which the anti-inflammatory activity of novel macrolide compounds could be measured. Finally, on the basis of *in vitro* and *in vivo* experiments, an hypothesis for the possible mechanism of inhibition of lung neutrophil infiltration was proposed.
Methods

Mice.

Studies were performed on 10 week old male BALB/cJ mice (Charles River, Lyon, France). Mice were kept on wire mesh floors with irradiated maize granulate bedding (Scobis Due, Mucedola, Settimo Milanese, Italy) and maintained under standard laboratory conditions (temperature 23-24°C, relative humidity 60±5%, 15 air changes per hour, artificial lighting with circadian cycle of 12 hours). Pelleted food (VRF-1, Altromin, Charles River, Isaszag, Hungary) and tap water were provided *ad libitum*.

All procedures on animals were approved by the Ethics committee of GlaxoSmithKline Research Centre Zagreb Limited, and performed in accordance with the EEC Council Directive 86/609.

Materials: chemicals, antibodies and drugs.

LPS from *Escherichia coli* serotype 0111:B4 was obtained from Sigma Chemical Co. (St Louis, MO, US). Luminex kits and antibodies for enzyme-linked immunosorbent assay (ELISA) were purchased from R&D Systems (Minneapolis, MN, US). Azithromycin was from PLIVA Inc. (Zagreb, Croatia) and clarithromycin was from Spectrum Chemical Mfg. Corp. (Gardena, CA, US). All other reagents, if not indicated otherwise, were from Sigma Chemical Co. (St Louis, MO, US).

LPS-induced pulmonary neutrophilia.

Experimental pulmonary neutrophilia was induced as described earlier (Ivetic, et al., 2006). Briefly, mice, under light anesthesia, were instilled intranasally with 2 μg LPS from *E. coli*/60 μl PBS. Vehicle, clarithromycin and azithromycin were administered orally by gavage 4 h before intranasal challenge with LPS. For administration, macrolides were first dissolved in dimethysulfoxide (DMSO) and then diluted with 0.5% (w/v) methyl-cellulose [final concentration of DMSO was 5% (v/v)]. Azithromycin was further solubilized by addition of
an equimolar quantity of citric acid. Solutions obtained were applied orally in a volume of 20 ml/kg (b.w.). Firstly, macrolides were tested at doses of 150, 300 and 600 mg/kg to determine the lowest effective dose at which compounds statistically significantly decreased total cell and neutrophil numbers in bronchoalveolar lavage fluid 24 h after challenge with LPS. Based on the results obtained, a dose of 600 mg/kg was used in subsequent time-course experiments.

**Bronchoalveolar lavage and determination of total and relative cell number in bronchoalveolar lavage fluid (BALF).**

Immediately prior (0 h) or at various time points after LPS application, the animals were euthanized by an intraperitoneal overdose of Thiopental® (Inresa Arzneimittel GmbH, Freiburg, Germany). After preparation and cannulation of tracheas, bronchoalveolar lavage was performed with phosphate buffered saline (PBS) in a total volume of 1 ml (0.4, 0.3 and 0.3 ml). Following bronchoalveolar lavage, lungs were excised and fixed in 10% buffered neutral formalin fixative.

The bronchoalveolar lavage samples were centrifuged (4°C, 100g, 5 min), the pellet of cells resuspended in an equal volume of fresh PBS and used for total and differential cell counts. Total number of cells in BALF was counted with a hematological analyzer (Sysmex SF 3000, Sysmex Co., Kobe, Japan). Percentages of neutrophils and macrophages were determined by morphological examination of at least 200 cells on smears prepared by cytocentrifugation (Cytospin-3, Thermo Fisher Scientific Inc., Pittsburgh, PA, US) and stained with Kwik-Diff staining set (Thermo Fisher Scientific Inc., Pittsburgh, PA, USA). Number of neutrophils (and macrophages) in BALF was calculated for each sample according to the formula:

Number of neutrophils = Total number of cells x (Neutrophil percentage / 100%)

**Histopathological examination of lungs.**
After collection of BALF, lungs from all animals were formalin-fixed, paraffin-embedded in toto, cut at 3 \( \mu \)m and stained routinely with hematoxylin-eosin. For each lung specimen, neutrophilic granulocyte infiltration into peribronchial, periarterial and perivenular areas as well as in interstitium and alveolar space as well as interstitial and alveolar areas, was examined by an observer blinded to the experimental design and was graded according to the following criteria:

0: No granulocytes

1: Few scattered granulocytes

2: Larger aggregates of granulocytes

3: Marked accumulation of granulocytes

In borderline cases, an intermediate grade was used (0–1, 1–2, 2–3), extending the scoring to a total of seven grades.

Preparation of lung homogenates for determination of inflammatory mediators and adhesion molecules.

In separate groups of mice, non-lavaged lungs were collected for determination of the concentrations of inflammatory mediators. Lungs were homogenized on ice in PBS with protease inhibitors [1 \( \mu \)g/ml leupeptin, 2 \( \mu \)g/ml aprotinin, 1 \( \mu \)g/ml pepstatin, and 17 \( \mu \)g/ml phenylmethylsulphonyl fluoride]; 4 ml of PBS with protease inhibitors was added per gram of lung tissue. Homogenates were centrifuged (4°C, 2500g, 15 min) and stored at –80°C until analysis.

Determination of protein concentration.

Protein concentration in lung homogenates was determined by BCA Protein Assay (Thermo Fisher Scientific Inc., Waltham, MA, US) according to the manufacturer's recommendation.

Measurement of inflammatory mediators in lungs.
Samples were analyzed using xMAP technology, which enables simultaneous measurement of multiple biomarkers. Concentrations of GM-CSF, IFNγ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-13, IL-17, CCL2 (JE), CXCL1 (KC), CXCL2 (MIP-2), TNF-α were determined using Fluorokine MAP multiplex kit (R&D Systems, Minneapolis, MN, US) according to the manufacturer’s protocol. Briefly, 50 μl of samples were incubated with antibody-coated microparticles for 3 h at room temperature. Afterwards, washed beads were incubated with biotinylated detection antibody cocktail for 1 h at room temperature, washed and incubated for 30 min with streptavidin-phycoerythrin conjugate. After the final wash the microparticles were resuspended in buffer and analyzed with the Luminex 200™ (Luminex, Austin, TX, US) and STarStation software v2.3 (Applied Cytometry Systems, Sacramento, CA, US) using a five-parameter-logistic-curve fitting.

Myeloperoxidase (MPO) concentration in lung homogenates was determined by Mouse MPO ELISA kit (Hycult Biotechnology b. v., Uden, The Netherlands) according to the manufacturer’s recommendations. Optical density was measured at 450 nm using a microplate reader (SpectraMax 190, Molecular Devices Corporation, Sunnyvale, CA, US). MPO concentration was determined by interpolation from standard curves with SoftMax Pro v 4.3.1 software (Molecular Devices Corporation, Sunnyvale, CA, US).

Concentration of analytes in lung homogenates was further normalized to protein concentration in the samples and expressed as pg of analyte per mg of protein.

**Measurement of adhesion molecules in lungs.**

Concentrations of sE-selectin, soluble intracellular adhesion molecule 1 (sICAM-1) and soluble vascular adhesion molecule 1 (sVCAM-1) were determined using Mouse cardiovascular disease panel LINCOplex kit (LINCO Research, St. Charles, MO, US) according to the manufacturer’s protocol. Briefly, 25 μl of samples were incubated with antibody-coated microparticles overnight at +4°C. Afterwards, washed beads were incubated...
with biotinylated detection antibody cocktail for 1 h at room temperature followed by a 30 min incubation with streptavidin-phycoerythrin conjugate. After the final wash the microparticles were resuspended in sheath fluid and analyzed with the Luminex 200™ (Luminex, Austin, TX, US) and STarStation software v2.3 (Applied Cytometry Systems, Sacramento, CA, US) using a five-parameter-logistic-curve fitting. Concentration of analytes in lung homogenates was further normalized to protein concentration in the samples and expressed as pg or ng of analyte per mg of protein.

**Cells.**

The murine monocyte/macrophage cell line, J774.2 was obtained from the European Collection of Cell Cultures (Porton Down, UK), while murine lung epithelial cells, MLE 12 were from American Type Culture Collection (Manassas, VA, US). J774.2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Gibco, Carlsbad, CA, US) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (BioWest, Nuaillé, France). MLE 12 cells were maintained in bronchial epithelial basal medium (BEBM; Lonza, Walkersville, MD, US) supplemented with growth factors (bovine pituitary extract, hydrocortisone, hEGF, insulin, and transferrin), gentamicin and amphotericin-B (all provided as BEGM SingleQuots, Lonza), and 2% FBS.

**Cell stimulation and inhibition.**

For all in vitro experiments macrolides were dissolved in DMSO at a concentration of 50 mM and a series of two fold dilutions in DMSO were prepared. Macrolide DMSO stock solutions were diluted 1000 fold in cell culture medium to desired concentrations. Therefore, DMSO concentration was 0.1% in all samples.

J774.2 cells were seeded in a 24-well plates in DMEM with 10% FBS at a concentration of $3 \times 10^5$ cells per well. The next day, cells were pre-incubated with macrolides
for 2 h and stimulated overnight with 1 μg/ml LPS from *E. coli* serotype 0111:B4. At the end of the incubation period, supernatants were collected and stored at -20°C until assayed.

MLE 12 cells were seeded in 24-well plates in supplemented BEBM at a concentration of 2.5×10⁵ cells per well and grown to confluence. Afterwards cells were pre-incubated with macrolides in BEBM supplemented only with 2% FBS for 2 h and stimulated overnight with 10 ng/ml LPS from *E. coli* serotype 0111:B4. At the end of the incubation period, supernatants were collected and stored at -20°C until assayed. Optimal concentrations of LPS for stimulation of J774.2 and MLE were established in preliminary experiments.

**ELISA.**

Cytokine concentrations were determined in cell culture supernatants by sandwich ELISA using capture and detection antibodies according to the manufacturer’s instructions. Sensitivity of the assay was 0.1 pg/ml for CCL2, 12.2 pg/ml for CXCL1, 154.1 pg/ml for CXCL2, 4.6 pg/ml for GM-CSF, 20.2 pg/ml for IL-1β, 5.5 pg/ml for IL-6, and 25.5 pg/ml for TNF-α. Optical density was measured at 450 nm using a microplate reader (SpectraMax 190). Concentration of cytokines was determined by interpolation from standard curves with SoftMax Pro v 4.3.1 software (Molecular Devices Corporation).

**Statistical analysis.**

All values are presented as means ± S.E.M.. To define statistically significant differences among vehicle-treated and macrolide-treated mice after LPS challenge, the data were subjected to two way ANOVA followed by Bonferroni post test using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, US). To define statistically significant differences between vehicle-exposed and macrolide-exposed cells in vitro, the data were subjected to one way ANOVA followed by Dunnett's post test using GraphPad Prism. The level of significance was set at *p*<0.05 in all cases.
Results

Effect of macrolides on inflammatory cell accumulation in airways and lungs.

The lowest effective oral does at which azithromycin and clarithromycin statistically significantly decreased total cell and neutrophil numbers in bronchoalveolar lavage fluid, 24 h after challenge with LPS, is 600 mg/kg (b.w.) (Fig 1A and B). Therefore, this dose was used in subsequent time-course experiments, in which effects of macrolides on the inflammatory cascade leading to neutrophil infiltration into the lungs was assessed following intranasal LPS challenge.

An increase in the number of inflammatory cells in airways (BALF) was observed at 6 h and progressed until 24 h following LPS instillation. As expected, infiltrated cells were neutrophils, while macrophage numbers did not change significantly. Macrolide treatment almost completely blocked initial inflammatory cell accumulation in BALF at 6h, and significantly decreased total cell and neutrophil numbers in BALF (~50%) at 24 h after LPS challenge (Fig. 2A and B).

In order to quantitatively measure neutrophil levels in the lungs, the concentration of a neutrophil-specific enzyme, MPO, in lung homogenates was determined. MPO concentration in lungs reached maximal levels already 4 h after LPS challenge and remained increased for at least 24 h (Fig. 3). The effect of both tested macrolides on LPS-induced increase of MPO was even more striking than their effects on inflammatory cell accumulation in BALF (Fig. 3). Surprisingly, MPO concentration was significantly increased 4 h after azithromycin, but not clarithromycin administration to naïve animals.

Histopathological examination of the lungs confirmed accumulation of neutrophils around arteries, veins and bronchi and in alveoli of LPS treated animals (Fig. 4B). Treatment with azithromycin decreased the number of neutrophils around arteries and bronchi (Fig. 4C). Additionally, azithromycin postponed and reduced the perivein and intra-alveolar
accumulation of neutrophils. Clarithromycin had a similar effect on neutrophil accumulation as azithromycin (Fig. 4D).

**Effect of macrolides on inflammatory mediators in lungs.**

Following the demonstration that macrolides efficiently inhibit LPS-induced pulmonary infiltration of inflammatory cells, their effects on inflammatory mediators were evaluated. In a preliminary experiment, LPS-mediated increases in GM-CSF, IL-1β, IL-6, CCL2 (JE), CXCL1 (KC), CXCL2 (MIP-2) and TNF-α in lung homogenates were observed, while IFNγ, IL-2, IL-4, IL-5, IL-10, IL-12p70, IL-13 and IL-17 were below the limit of detection (data not shown) and therefore not measured in the subsequent experiments.

Azithromycin and clarithromycin did not inhibit the production of two major neutrophil chemoattractants in mice (CXCL1 and CXCL2) which were strongly induced immediately following LPS challenge, peaked 4 h post challenge and gradually decreased afterwards (Fig. 5A and B). Similar to the effects observed on neutrophil infiltration into lungs, azithromycin pretreatment increased CXCL1 concentration in naïve mice (at 0 h). On the other hand, azithromycin as well as clarithromycin, decreased LPS-induced TNF-α, IL-6, GM-CSF, CCL2 and IL-1β levels. Whereas IL-6 concentration was only slightly decreased between 4 and 8 h after LPS challenge (Fig. 5D), TNF-α concentration was significantly decreased (for ~50%) at all time points after LPS challenge except at 24 h when TNF-α levels had almost returned to baseline (Fig. 5C). The most striking effect of macrolides was seen on GM-CSF and CCL2: both azithromycin and clarithromycin almost completely inhibited LPS-induced release of these two cytokines, which peaked at 4 and 8 h after LPS challenge, respectively (Fig. 5E and F). In contrast to other measured inflammatory mediators, IL-1β concentration increased at 4 h but remained elevated even 24 h following LPS challenge, and it was significantly inhibited by both compounds (Fig. 5G).

**Effect of macrolides on adhesion molecules in lungs.**
In addition to modulation of production of cytokines, macrolides could affect neutrophil infiltration into the lungs also by decreasing expression of adhesion molecules. Therefore, concentrations of several adhesion molecules were determined in lung homogenates. LPS-induced increase in sE-selectin concentration between 4 and 8 h after challenge was strongly decreased by both tested macrolides (Fig. 6A). In contrast to sE-selectin, a continuous increase in sICAM-1 concentration was observed up to 24 h following LPS challenge, and the macrolide effect on sICAM-1 concentration was observed only at the latest time point investigated (Fig. 6B). On the other hand, sVCAM-1 concentration in lung homogenates was not changed following LPS administration (Fig. 6C).

**Effect of macrolides on cytokine production by LPS-stimulated J774.2 and MLE 12 cells.**

The results presented so far indicate that macrolides efficiently suppress LPS-induced neutrophil accumulation, which could be explained by the decreased production and/or release of inflammatory mediators and adhesion molecules. The main mouse lung cell types that express TLR4 and co-receptors required for LPS recognition, and seem to be responsible for initiation of inflammation in lungs following LPS challenge, are macrophages and epithelial cells (Martin, 2000; Saito, et al., 2005). Therefore, in order to validate the biomarkers detected *in vivo*, we examined the ability of macrolides to suppress LPS-induced cytokine production from lung epithelial cells, MLE 12, and the monocyte-macrophage cell line, J774.2.

In the epithelial cell line, MLE 12, macrolides did not influence production of any of the LPS-stimulated cytokines (IL-6, GM-CSF, CXCL1, and CCL2; Figure 7). On the other hand, in the macrophage cell line, J774.2, macrolide pre-treatment dose-dependently suppressed the LPS-induced production of IL-1β, IL-6 and GM-CSF (Fig. 8), while having no inhibitory effect on LPS-stimulated production of TNF-α, CCL2, CXCL1, and CXCL2 (data
not shown). The viability of cells was not affected by treatment with macrolides as no significant increase in adenylate kinase was observed in supernatants at the end of incubation (data not shown).
Discussion

Neutrophil infiltration is prominent in many lung diseases but its resistance to corticosteroid therapy prompted a search for drugs that inhibit neutrophilic inflammation (Barnes, 2007). Human and animal investigations, including our experimental findings presented here using cell counts, histology and MPO determinations, show that macrolide treatment efficiently suppresses neutrophil-dominated pulmonary inflammation. However, the mechanism is not entirely clear, so we studied the inflammatory cascade leading to lung neutrophil accumulation following LPS challenge. We aimed to identify potential biomarkers of macrolide anti-inflammatory activity, subsequently applicable to a relatively simple in vitro test to profile non-antibacterial, anti-inflammatory macrolide compounds.

The dose showing anti-inflammatory effects in this animal model (600 mg/kg) is higher than those exerting therapeutic effects in humans (ranging from 250 mg every other day to 500 mg qd). However, to achieve therapeutic effects in chronic inflammatory diseases, macrolides have to be administered for long periods of several months or even years (Crosbie and Woodhead, 2009). This may explain why, in our acute animal model of inflammation, a single high dose is needed. Moreover, Criqui et al. (2000) showed that even in clinical settings, short term treatment with azithromycin (500 mg on day 1 followed by 250 mg daily for 3 days) is not sufficient to reduce an acute inflammatory response induced by ozone.

Within 2 h of intranasal LPS challenge, lung inflammation is characterized by production of three mediators: CXCL1, CXCL2, and TNF-α. Surprisingly, macrolides did not inhibit production of the major murine neutrophilic chemoattractants, CXCL1 and CXCL2, either at this early or at later time-points. Moreover, macrolides failed to inhibit LPS-induced production of these chemokines in epithelial and macrophage cell lines in vitro. In contrast, production of TNF-α was weakly, but statistically significantly decreased by azithromycin and clarithromycin (33 and 54%, respectively, at peak levels, 4 h after LPS challenge). This
weak inhibitory activity of the macrolides was not corroborated by *in vitro* findings. Since various cell types contribute to lung inflammation, the inhibition of TNF-α production observed *in vivo* could be the consequence of effects on cell type(s) other than the macrophages and epithelial cells studied. On the other hand, lung neutrophil infiltration was shown to be intact after i.n. LPS administration to TNF-α deficient mice (Schnyder-Candrian, et al., 2005). Moreover, intratracheal injection of TNF-α into healthy mice induces only very mild lung inflammation (Ulich, et al., 1991 and our unpublished results). Therefore, macrolide-mediated inhibition of TNF-α is unlikely to be a prerequisite for inhibition of lung neutrophil accumulation.

That inflammatory mediators produced early after LPS challenge (CXCL1, CXCL2 and TNF-α) are uninvolved in macrolide-induced decreases in neutrophil accumulation agrees with the findings of Sanz et al. (2005). They reported that erythromycin treatment reduced BALF neutrophil counts and lung tissue MPO activity in LPS-challenged rats without effects on CXCL2 (MIP-2) and TNF-α levels in BALF.

In contrast to effects on early inflammatory mediators, macrolide treatment significantly attenuated increases in IL-6, GM-CSF, CCL2 and IL-1β concentrations from 4 h after LPS challenge. Azithromycin and clarithromycin weakly inhibited - like TNF-α - IL-6 production (~30%) between 4 h and 8 h after LPS challenge *in vivo*, and IL-6 production by macrophages *in vitro*. IL-6 plays a dual role in LPS-induced neutrophil accumulation, possessing both anti- and pro-inflammatory properties. LPS administration to IL-6−/− mice induced significantly higher neutrophilic responses and increases in TNF-α and CXCL2 (MIP-2) in comparison to responses in wild-type mice (Xing, et al., 1998). Yet IL-6 was required for leukocyte migration into the lungs, and for the neutrophil to monocyte recruitment transition during inflammation (Kaplanski, et al., 2003). Therefore, mild IL-6 inhibition induced by macrolides probably does not directly affect neutrophil accumulation,
but could ameliorate later stages of the inflammatory response to LPS, when transition to monocyte accumulation occurs.

Although macrolides inhibited TNF-α and IL-6 only moderately, they almost completely blocked LPS-induced GM-CSF generation in vivo. GM-CSF production by macrophages in vitro was also inhibited. GM-CSF is a survival factor for neutrophils, and its neutralization by anti-GM-CSF antibodies suppresses LPS-induced neutrophilic inflammation in lungs (Bozinovski, et al., 2004). Moreover, anti-GM-CSF antibody treatment did not influence expression of CXCL2 (MIP-2), and reduced peak TNF-α levels only by 37%, in keeping with effects of macrolides in our study. Thus, macrolides by inhibiting GM-CSF could reduce neutrophil lifespan, increase apoptosis and subsequently reduce their number in LPS-challenged lungs. This suggestion is strengthened by findings that the number of apoptotic granulocytes in blood of healthy volunteers was significantly increased after 3-day dosing with azithromycin (500 mg/day, p.o.) (Culic, et al., 2002). Moreover, macrolides decreased neutrophil survival in vitro through inhibition of GM-CSF release from activated human airway epithelial cells (Yamasawa, et al., 2004). Interestingly, in our study, macrolides failed to inhibit GM-CSF production by mouse epithelial cells, implying a species-dependent, differential cell target for the anti-inflammatory effects of macrolides, currently under investigation in our laboratory. Nevertheless, inhibition of GM-CSF production by macrolides could well account for reduced LPS-induced cellular accumulation into mouse lungs.

Azithromycin and clarithromycin also dramatically (73% and 96%, respectively) suppressed lung CCL2. Although neutrophils do not express the CCL2 receptor, CCR2, CCL2 still seems to be important for lung neutrophil accumulation. Treatment of mice with anti-CCL2 antibodies reduced both monocyte and neutrophil recruitment during pulmonary Cryptococcus neoformans infection (Huffnagle, et al., 2000). Moreover, intratracheal challenge with CCL2 and LPS strongly augmented neutrophil influx into alveolar spaces, in
comparison to challenge with LPS alone (Maus, et al., 2003). Therefore, inhibition of CCL2 production by macrolides could contribute to reduced neutrophil influx into LPS-challenged mouse lungs. Inhibition of CCL2 production was not observed in LPS-stimulated macrophages and epithelial cells in vitro. This contradictory result can be explained by the finding that CCL2 production is increased both by LPS and the induced cytokines, TNF-α and IL-1β (Standiford, et al., 1991). Consequently, it is likely that the inhibition of CCL2 production after macrolide treatment in vivo was secondary to reduction of TNF-α and IL-1β levels, while in epithelial and macrophage monocultures in vitro, TNF-α and thereby CCL2 production remained unchanged.

In contrast to other inflammatory mediators, the pulmonary IL-1β increased between 2h and 4h, remaining elevated until 24h after LPS challenge. IL-1β alone is sufficient to induce substantial lung neutrophil accumulation (Lappalainen, et al., 2005; Ulich, et al., 1991). Therefore, its inhibition by macrolides in vivo and in alveolar macrophages in vitro, seems to be a major mechanism for reduction of cellular influx into lungs. IL-1β was, in fact, the only significantly decreased pro-inflammatory cytokine in BALF after 12-weeks azithromycin treatment of COPD patients (Hodge, et al., 2008). These data further support the validity of IL-1β as a biomarker of macrolide anti-inflammatory activity in the lungs.

Since the spectrum of cytokine inhibition in vivo was mirrored in monocyte/macrophages, but not in epithelial cells in vitro, it is likely that alveolar macrophages are the target cells for macrolide activity in our in vivo model. Nevertheless, macrolides may also act on other pulmonary cell types, either directly inhibiting LPS signaling or that of pro-inflammatory cytokines.

Macrolide treatment also reduced levels of two upregulated adhesion molecules, sE-selectin and sICAM-1. Inhibition of adhesion molecule expression by macrolides was reported in a similar animal model (Sanz, et al., 2005) as well as clinically (Hillis, et al.,
However, since these adhesion molecules are induced by both LPS and pro-inflammatory cytokines, such as TNF-α and IL-1β, the observed inhibition may be indirect, through effects of macrolides on cytokines.

Interestingly, azithromycin, but not clarithromycin, increased lung CXCL1 and MPO concentrations at the time of challenge. This pro-inflammatory activity of azithromycin corresponds to a biphasic effect reported previously (Culic, et al., 2002; Shinkai, et al., 2006). Azithromycin (500 mg/day for 3 days) initially stimulated blood neutrophil degranulation and oxidative burst to particulate stimuli in healthy volunteers a response followed by a continual fall in IL-8, GRO-α, and IL-6 concentrations, a delayed down-regulation of the oxidative burst and an increase in apoptosis of neutrophils (Culic, et al., 2002). Similarly, azithromycin and clarithromycin increased IL-8 production by normal human bronchial epithelial cells at 24 h after LPS stimulation, but after prolonged incubation (for 5 days), decreased IL-8 levels in comparison to LPS alone (Shinkai, et al., 2006). The early pro-inflammatory activity of azithromycin in our model in vivo, however, is unrelated to its anti-inflammatory effects on the monitored inflammatory parameters, as these were generally comparable to those of clarithromycin.

In conclusion, the effective suppression by macrolide antibiotics of pulmonary neutrophil-dominated inflammation appears to involve inhibition of GM-CSF and IL-1β production by alveolar macrophages. Measurement of these mediators in LPS-stimulated macrophages in vitro could represent a simple test for novel anti-inflammatory macrolides. In addition, CCL2 and sE-selectin show potential as biomarkers for macrolide anti-inflammatory activity in the lungs. If GM-CSF and IL-1β are indeed major targets for macrolides in reducing murine lung neutrophilia, inflammation induced by one or both of these mediators should be resistant to macrolides. This will be addressed in future studies. In addition, in vitro
mechanistic studies will be extended to human bronchial epithelial cells and monocyte-derived macrophages.
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Footnotes

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Reprint requests:

Martina Bosnar,
GlaxoSmithKline Research Centre Zagreb Limited,
Prilaz baruna Filipovića 29,
HR-10000 Zagreb, Croatia
Email address: martina.z.bosnar@gsk.com

M.B. and B.B. equally contributed to this work.
Legends for Figures

**Fig. 1.** Total cell (A) and neutrophil (B) number in BALF 24 h after LPS challenge. Azithromycin or clarithromycin were administered orally 4 h prior LPS challenge at doses of 150, 300 and 600 mg/kg. Data are presented as means ± S.E.M.. Asterisk represents p<0.05, Kruskal-Wallis test; Dunn's multiple comparison test.

**Fig. 2.** Total cell (A), neutrophil (B) and macrophage (C) number in BALF at different time points after LPS challenge (solid squares). Azithromycin (open triangles) or clarithromycin (open circles) were administered orally 4 h prior LPS challenge at a dose of 600 mg/kg. Data are presented as means ± S.E.M.. Asterisk represents p<0.05, Two-way ANOVA; Bonferroni post test.

**Fig. 3.** MPO concentration in lung homogenates at different time points after LPS challenge (solid squares). Azithromycin (open triangles) or clarithromycin (open circles) were administered orally 4 h prior LPS challenge at a dose of 600 mg/kg. Data are presented as means ± S.E.M. Asterisk represents p<0.05, Two-way ANOVA; Bonferroni post test.

**Fig. 4.** Representative photomicrographs of lung tissue (hematoxylin and eosin, ×400) at 24 h after challenge with saline (A) or 2 µg of lipopolysaccharide (LPS) (B, C and D). Arrows indicate granulocyte infiltration. The PBS-challenged group showed normal lung histology (A). In the LPS-challenged (2 µg/animal intranasally), vehicle-treated group, marked infiltration of granulocytes into the lung was observed (B). Oral azithromycin (C) or clarithromycin (D) treatment (600 mg/kg) decreased infiltration of neutrophils into lung tissue in comparison to the LPS-challenged group. Internal scale bars, 100 µm.

**Fig. 5.** Concentration of CXCL1 (A), CXCL2 (B), TNF-α (C), IL-6 (D), GM-CSF (E), CCL2 (F) and IL-1β (G) in lung homogenates at different time points after LPS challenge (solid squares). Azithromycin (open triangles) or clarithromycin (open circles) were administered.
orally 4 h prior LPS challenge at a dose of 600 mg/kg. Data are presented as means ± S.E.M.
Asterisk represents p<0.05, Two-way ANOVA; Bonferroni post test.

**Fig. 6.** Concentration of sE-selectin (A), sICAM-1 (B) and sVACM-1 (C) in lung homogenates at different time points after LPS challenge (solid squares). Azithromycin (open triangles) or clarithromycin (open circles) were administered orally 4 h prior LPS challenge at a dose of 600 mg/kg. Data are presented as means ± S.E.M.. Asterisk represents p<0.05, Two-way ANOVA; Bonferroni post test.

**Fig. 7.** Effect of macrolides on CXCL1 (A), IL-6 (B), GM-CSF (C), and CCL2 (D) production by LPS-stimulated MLE 12 cells. Cells were pre-incubated with compounds for 2 h following an overnight LPS stimulation. Data are presented as means ± S.E.M. from three different experiments. Asterisk represents p<0.05, one way ANOVA; Dunnett's post test.

**Fig. 8.** Effect of macrolides on IL-6 (A), GM-CSF (B), and IL-1β (C) production by LPS-stimulated J774.2 cells. Cells were pre-incubated with compounds for 2 h following an overnight LPS stimulation. Data are presented as means ± S.E.M. from four different experiments. Asterisk represents p<0.05, one way ANOVA; Dunnett's post test.
Figure 1
Figure 3
Figure 5
Figure 6

(A) Graph showing sE-selectin (pg/mg protein) over hours post challenge.

(B) Graph showing sICAM-1 (pg/mg protein) over hours post challenge.

(C) Graph showing sVCAM-1 (pg/mg protein) over hours post challenge.
Figure 7
Figure 8